IMMUNOLOGICAL STUDY OF GLUTAMATE SYNTHASE AND ITS SUBUNITS

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1. Introduction

Glutamate synthase is a flavin-containing glutamine amidotransferase (EC 2.6.1.53) that catalyzes the transfer of the amide nitrogen of glutamine to 2-oxoglutarate [1–3]. The enzyme also catalyzes a reaction in which ammonia is substituted for glutamine [2–4]. Glutamate synthase has been purified to homogeneity from *Escherichia coli* [1,3], *Aerobacter aerogenes* [2] and *Bacillus megaterium* [5]. The enzyme isolated from these bacteria is composed of two non-identical subunits. The large subunit (MW 135 000–175 000) serves as the glutamine binding site [2,3], while the function of the small subunit (MW ~ 50 000) is still uncertain.

In this paper, we describe an immunological study of glutamate synthase and its subunits. The objective of this research is to study immunological relatedness of glutamate synthase and its subunits in enterobacteria compared to the properties of the enzyme from non-enterobacterial cells.

2. Materials and methods

2.1. *Strains and cultures*

Klebsiella aerogenes KM-270 (a GDH⁻ mutant) was generously provided by Dr R. A. Bender (Massachusetts Institute of Technology). The culture medium of Brenchley et al. [6] was used. *K. pneumonia*, *Escherichia coli* and *Bacillus subtilis* were cultured in a similar medium. *Salmonella typhimurium* was cultured in the same medium with addition of 50 mg adenine and 1 mg thiamine/l.

2.2. *Purification of glutamate synthase from* Klebsiella aerogenes *and separation of the subunits*

Glutamate synthase was purified to homogeneity from 320 g of *K. aerogenes* cells as previously described [3]. The enzyme's homogeneity was evaluated by analytical polyacrylamide gel electrophoresis, sodium dodecyl sulfate gel electrophoresis and immunodiffusion. The homogenous enzyme had a specific activity of 12.1 units/mg protein at room temperature.

Subunits were separated on an Ultrogel AcA 34 column (1.5 × 130 cm) in the presence of 0.3% SDS [7,8]. SDS was removed by chromatography on a Dowex 1 column [9]. Subunit separation was also performed by polyacrylamide gel electrophoresis in the presence of 0.1% SDS or 3 M urea (polyacrylamide 4%, gels 1 × 10 cm). Protein loaded on a gel was 440 μg. The concentrated subunits were mixed with an equal amount of glycerol and stored at –20°C.

2.3. *Immunochemical procedures*

2.3.1. Preparation of anti-glutamate synthase and the anti-subunits

New Zealand white rabbits were immunized with 1 nmol of the native enzyme or about 2 nmol of the subunits in Freund's complete adjuvant. Antisera were prepared as described in [10].

2.3.2. Immunodiffusion and immunoprecipitation

Ouchterlony immunodiffusion [11] was used.

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Quantitative precipitation was performed as described in [12].

2.3.3. Iodination of glutamate synthase

Glutamate synthase was iodinated using the chloramine T procedure [13]. In most cases 1 mCi of ^{125}I was used per 220 μg of protein. After gel filtration on Sephadex G-25 and dialysis, the iodinated enzyme was stored at −70°C.

2.3.4. Radioimmunoassay

Reaction mixtures of 0.44 ml contained antibody (14 ng anti-glutamate synthase, 0.48 μg anti-large subunit, 0.35 μg anti-small subunit), 0.03 μg (17 000 cpm) ^{125}I-glutamate synthase, 50 mM Tris-acetate, pH 7.6, 5% bovine serum albumin and competing antigen. After a 24-h incubation at 4°C an excess goat anti-rabbit γ-globulin was added. After another 24-h incubation at 4°C the precipitates were centrifuged, washed and counted for radioactivity.

3. Results and discussion

3.1. *Antigen purity*

Disc gel electrophoresis and immunodiffusion affirmed the homogeneity of glutamate synthase and the large and small subunits. The immunodiffusion patterns are shown in figs.1a, b and c. In each case only a single line of precipitation was detected.

3.2. *Cross-reactivity of glutamate synthase and its subunits from various bacteria with antibodies to* K. aerogenes *glutamate synthase and subunits*

Crude extracts from enterobacteria and *Bacillus subtilis* were tested against anti-glutamate synthase in Ouchterlony immunodiffusion. As seen in fig.1d glutamate synthase from various enterobacteria cross-reacted with anti-glutamate synthase to the *K. aerogenes* enzyme. Crude extract from *B. subtilis*, on the other hand, had no cross-reacting activity.

An antibody dilution of 1:500 (14 ng anti-glutamate synthase, 0.48 μg or 0.35 μg anti-large or anti-small subunit, respectively) was used. This amount of anti-glutamate synthase was able to precipitate about 60% of the radioactivity. The same dilution of anti-large subunit or anti-small subunit precipitated 7% and 5% of the radioactivity, respectively.

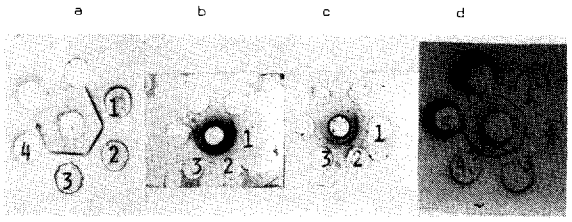

Fig.1. Immunodiffusion of glutamate synthase and its subunits with anti-glutamate synthase and anti-subunits. Well contents: (a) Center well 7.2 μg anti-glutamate synthase; 1,2,3 and 4: 14.6, 7.3, 2.9 and 1.5 μg purified glutamate synthase, respectively. (b) Center well 120 μg anti-large subunit; 1,2 and 3: 3.6, 1.4 and 0.7 μg denatured glutamate synthase, respectively. (c) Center well 90 μg anti-small subunit; 1,2 and 3: 3.6, 1.4 and 0.7 μg denatured glutamate synthase, respectively. (d) Center well 7.2 μg anti-glutamate synthase; outer wells contained crude extracts 1 from *K. aerogenes*, 0.26 mg; 2 from *K. pneumonia*, 0.36 mg; 3 from *E. coli*, 0.44 mg; 4 from *S. typhimurium*, 0.41 mg; 5 from *B. subtilis*, 0.29 mg.

Homologous glutamate synthase and crude extract from *K. aerogenes* bind to anti-glutamate synthase and inhibit precipitation of ^{125}I-glutamate synthase by 80% (fig.2A). Crude extracts from *K. pneumonia*, *E. coli* or *S. typhimurium* inhibited precipitation of ^{125}I-glutamate synthase by 18%, 16% and 10%, respectively.

We also studied cross-reactivity of the subunits from enterobacteria and *B. subtilis* with anti-subunits to *K. aerogenes* subunits. The crude extracts and ^{125}I-glutamate synthase were incubated in the presence of 0.4% SDS, dialysed and assayed for antibody–antigen reaction. The results in figs.2B and C clearly show extensive immunological relatedness of the subunits from enterobacteria. Neither the large nor the small subunit from *B. subtilis*, on the other hand, competed with the subunits of ^{125}I-glutamate synthase from *K. aerogenes* as do those from *K. pneumonia*, *E. coli* or *S. typhimurium*.

3.3. *Effect of anti-glutamate synthase on glutamine- and ammonia-dependent activities*

In general, the antigenic relatedness determined from immunoprecipitation and non-precipitated enzyme activity is in good agreement. The strongest decrease in glutamine-dependent activity was detected in the *K. aerogenes* and *K. pneumonia*

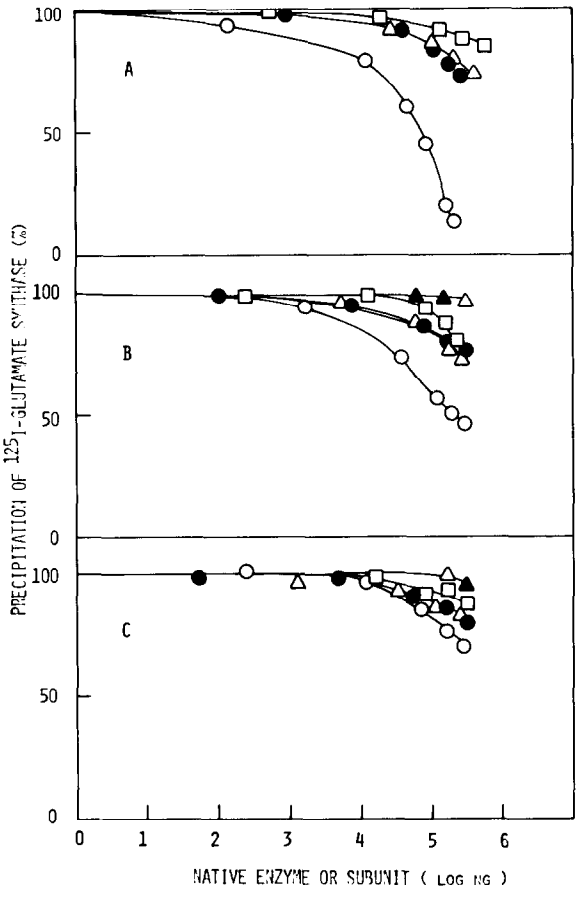

Fig.2. Inhibition of precipitation of ^{125}I-glutamate synthase-anti-glutamate synthase complex by bacterial crude extracts. Reaction conditions were as described under section 2. (A) Antibody was anti-glutamate synthase to *K. aerogenes* glutamate synthase. Competing antigen was from *K. aerogenes* (○–○); *K. pneumonia* (●–●); *E. coli* (△–△); *S. typhimurium* (□–□). (B) Anti-small subunit. *B. subtilis* (△–△). Otherwise the symbols of competing antigens are the same as in fig.2A. (C) Anti-large subunit. The symbols are the same as above.

activities and then in *E. coli* and *S. typhimurium* (table 1). The loss in enzyme activity was detected even when no precipitation of antibody–antigen complex was found. Ammonia-dependent activities were decreased during immunoprecipitation, as well.

Enterobacteria also showed similarity in quantitative immunoprecipitation (precipitin curves not shown). The maximal amounts of precipitation by anti-glutamate synthase to the *K. aerogenes* synthase

Table 1
Effect of anti-glutamate synthase on glutamine- and ammonia-dependent activities

Crude extract from	Remaining activity %	
	Gln-dependent	NH_3-dependent
K. aerogenes	32	64
K. pneumonia	37	67
E. coli	54	78
S. typhimurium	57	74
B. subtilis	86	92

The reaction mixtures contained about 2.5 mg crude extract protein and 6.7 ng antiserum

(7.2 μg protein) were 0.25 mg *K. aerogenes*, 0.25 mg *K. pneumonia*, 0.13 mg *E. coli* and 0.10 mg *S. typhimurium* protein. Equivalence zones were displayed in the same order and were between 0.12 and 0.01 enzyme units. These results indicate similarity in the number and affinity of antigenic determinants.

Several lines of evidence indicate the relatedness of glutamate synthases and the subunits from enterobacteria. (a) Single lines were detected in Ouchterlony immunodiffusion. (b) Inhibition of precipitation of ^{125}I glutamate synthase and its subunits by crude extract from enterobacteria. (c) Inhibition of glutamine- and ammonia-dependent glutamate synthase from different enterobacteria by antiglutamate synthase to the *K. aerogenes* synthase. (d) Quantity and profiles of immunoprecipitation were similar in enterobacteria.

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